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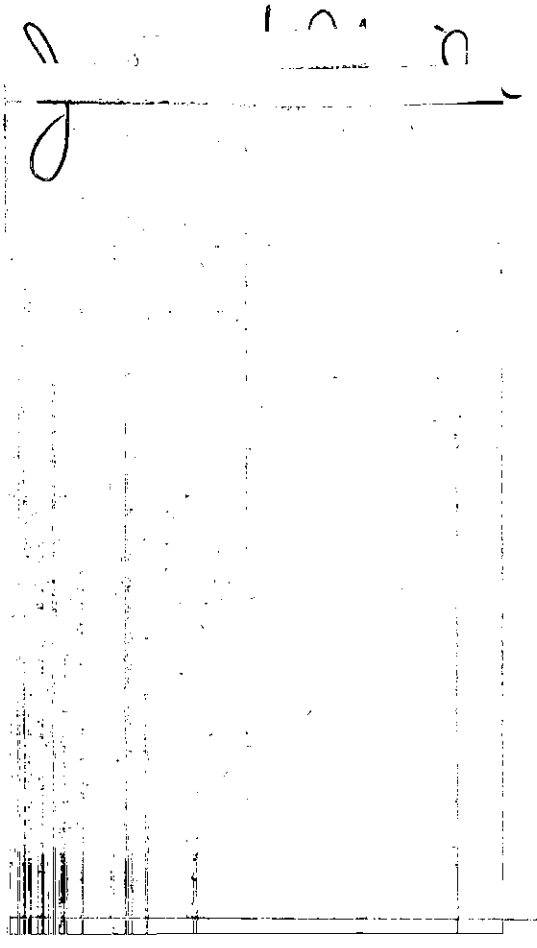

A MODEL SYSTEM FOR THE ALKALINE HYDROLYSIS OF RIBONUCLEIC ACID

A THESIS

Presented to

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by

James Herbert Guida

In Partial Fulfillment

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A MODEL SYSTEM FOR THE

ALKALINE HYDROLYSIS OF RIBONUCLEIC ACID

Approved:

Chairman

Date approved by Chairman: 3 VIII 64

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CHAPTER I

INTRODUCTION

Nucleic Acids

Ribonucleic acid (RNA) may be depolymerized by various hydrolytic means to give a mixture of nitrogenous bases, a pentose and inorganic phosphate.[1,2] Desoxyribonucleic acid (DNA) also can be degraded to a similar mixture of these fragments. These three groups of hydrolysis products are isolated in the ratio of one mole of the base mixture, to one mole of pentose to one mole of phosphate. Two major differences between the hydrolysates of RNA and DNA can be noted. Firstly, DNA produces adenine, guanine, cytosine and thymine as its mixture of bases whereas RNA gives adenine, guanine, cytosine and uracil. Secondly, and more germane to this work, DNA yields the pentose, D-2-desoxyribose whereas RNA gives D-ribose. D-2-desoxyribose and D-ribose are identical structurally except that the hydroxyl group in the 2 position of the sugar ring of D-ribose has been replaced by a hydrogen atom in D-2-desoxyribose.

The structure of the nucleic acid chain in DNA and RNA is made up of a backbone of the appropriate pentose, linked at the 3' - and 5' -positions by diesterified phosphoric acid moieties. The heterocyclic nitrogen

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[1] E. Chargaff and J. N. Davidson, in "The Nucleic Acids," Academic Press, New York, New York, 1955, pp. 1 - 30.

[2] R. F. Steiner and R. F. Beers, Jr., in "Polynucleotides," Elsevier Publishing Co., New York, New York, 1961, pp. 1 - 15.

bases are attached to the pentose by a glycosidic linkage at the 1'-carbon of the sugar ring (Figure 1). Despite the considerable structual similarities between RNA and DNA noted above, the two substances exhibit a marked difference in reactivity to mild basic hydrolysis. If RNA is subjected to mild alkaline hydrolysis, it is readily degraded to fragments called nucleotides, which would be considered monomers if compared to a simple polymeric system.[3] These nucleotides are phosphate monoesters in which the phosphoric acid fragment is linked to the sugar ring at the 2'- or 3'-position and the nitrogen base is left unaffected (Figure 1). DNA, however, is stable to similar basic hydrolysis as are most diesters of phosphoric acid.[4] The reason for this profound difference in reactivity between DNA and RNA lies at the 2'-hydroxyl group which DNA lacks. To understand this fully it is necessary to go back to research performed upon other phosphate esters.

Historical Background

In 1935 Bailly and Gaumé showed that whereas α-glyceryl phosphate is stable to alkaline hydrolysis, α-glyceryl methyl phosphate reacts to give methanol and a mixture of α- and β-glyceryl phosphates. No methyl phosphate was obtained.[5] Bauer and Kates reported

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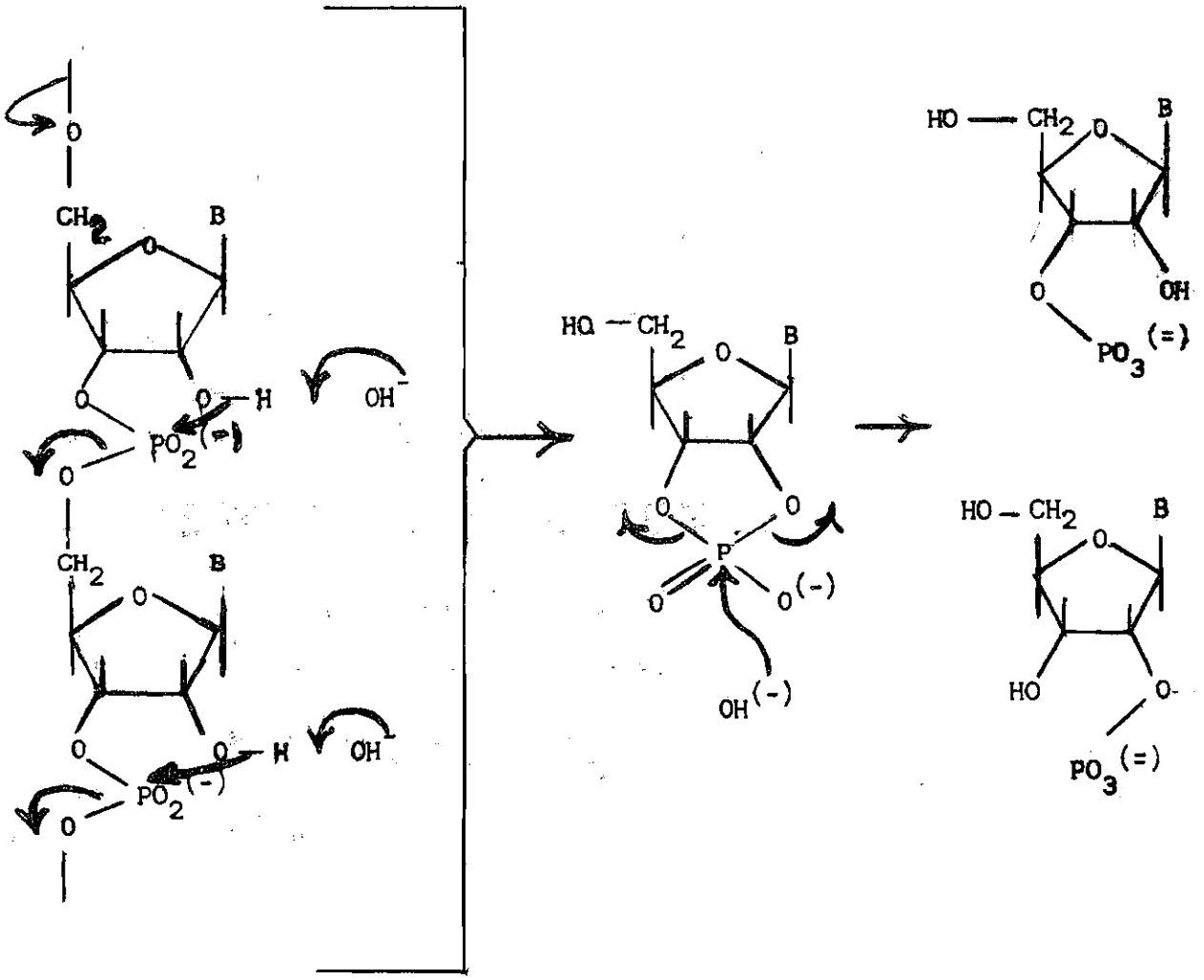
[3] In nucleosides, nucleotides and nucleic acids primed numbers refer by convention to positions on the sugar residue; unprimed numbers refer to positions on the nitrogen base.

[4] G. Kosolapoff, in "Organophosphorus Compounds," John Wiley and Sons, Inc., New York, New York, 1950, p. 233.

[5] O. Bailly and J. Gaumé, Bull. soc. chim. France, 2, 354 (1935).



A Ribonucleic Acid

A Cyclic Nucleotide Intermediate

A Mixture of 2' and 3' Nucleotides

Figure 1. Hydroxide Ion-catalyzed Hydrolysis of Ribonucleic Acid. B Represents Adenine, Guanine, Cytosine or Uracil.

similar results with α-glyceryl cholyl phosphate in which the only phosphates recovered were α- and β-glyceryl phosphates.[6] In an attempt to account for the unusual reactivity of the α-glyceryl methyl phosphate to base and suspecting that the β-hydroxyl was involved, Bailly and Gaumé prepared β-methoxyethyl methyl phosphate and β-hydroxylethyl dimethyl phosphate. The β-methoxy triester reacted in base as expected to give β-methoxyethyl methyl phosphate diester which was resistant to further hydrolysis. The β-hydroxyethyl methyl diester, however, showed the same anomalous reactivity to base as the α-glyceryl methyl phosphate diester by giving β-hydroxyethyl phosphate and methanol. No methyl phosphate was obtained.[7]

In 1940 Verkade postulated the presence of a cyclic intermediate in the acid-catalyzed migration of the phosphoryl group of α-glycerol phosphoric acid.[8] Chargaff later showed by using P_{15}^{32} that this reaction is intramolecular when catalyzed by acids or by an appropriate enzyme.[9]

Fono in 1947 seems to have been the first to apply the results of Bailly's studies of the neighboring hydroxyl's role in the hydrolysis of phosphodiesters. He suggested the formation of a cyclic triester in the alkaline hydrolysis of glyceryl alkyl phosphates and then went on to suggest an analogous triester intermediate to explain the differing

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[6] E. Bauer and M. Kates, J. Biol. Chem., 185, 615 (1950).

[7] O. Bailly and J. Gaumé, Bull. soc. chim. France, 3, 1396 (1936).

[8] P. E. Verkade, J. C. Stoppelenburg, and W. D. Cohen, Rec. trav. chim., 59, 886 (1940).

[9] E. Chargaff, J. Biol. Chem., 144, 455 (1942).

reactivities of RNA and DNA.[10] Kumler and Eiler had invoked interactions between hydroxyls on glycerol and sugar rings with the phosphoryl residue to explain the enhanced acidity of these compounds when compared to phosphoric acid itself.[11] The structural implications of Fono's suggestion were described in a brilliant paper by Brown and Todd.[12] Earlier it had been shown that nucleotides isolated from dilute aqueous solution could be resolved into two isomers.[13,14,15] The suggested cyclic intermediates would be labile to attack by hydroxide ion with non-stereospecific ring opening to give a mixture of 2'- and 3'-positionally isomeric nucleotides.

Cohn and coworkers subjected RNA to a hydroxide ion-catalyzed hydrolysis in H_2O^{18}.[16] Phosphate in the product was shown to contain one O^{18} atom per atom of phosphorus indicating that the phosphorus-oxygen bond is broken exclusively, and that the cyclic intermediate was not a triester as suggested by Brown and Todd[12] and Fono.[10]

Evidence indicating the presence of cyclic intermediates in the ribonuclease-catalyzed hydrolysis of RNA was supplied by the dilatometric

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[10] A. Fono, Arkiv. Kemi. Mineral. Geol., 24A, No. 34, 14, 15 (1947).

[11] W. D. Kumler and J. J. Eiler, J. Am. Chem. Soc., 65, 2355 (1943).

[12] D. M. Brown and A. R. Todd, J. Chem. Soc., 52 (1952).

[13] C. E. Carter and W. E. Cohn, Fed. Proc., 8, 190 (1949).

[14] H. S. Loring, N. G. Bortner and L. Levy, J. Am. Chem. Soc., 72, 1471, 2811 (1950).

[15] W. E. Cohn, J. Am. Chem. Soc., 72, 1466 (1950).

[16] D. Lipkin, P. T. Talbert, M. Cohn, J. Am. Chem. Soc., 76, 2871 (1954).

study of this reaction by Vandendriessche. He observed an initial increase in the volume of the reaction solution followed by a sharp decrease.[17]

About the same time as Brown and Todd's suggestion of a cyclic intermediate in the alkaline hydrolysis of RNA, Markham and Smith succeeded in isolating a substance from a ribonuclease-catalyzed hydrolysis of RNA which they considered to be a cyclic intermediate.[18] Shortly thereafter they isolated materials from $BaCO_3$ hydrolysis of RNA[19,20] which were identical with the synthetic cyclic nucleotides prepared by Brown and Todd.[21] These cyclic nucleotides were found to be unstable to both alkaline and enzymatic hydrolysis, yielding a mixture of 2'- and 3'-monophosphate nucleotides from the basic reaction and the 3'-nucleotide exclusively from the enzymatic reaction.[22,22a]

At this point it seemed clear that RNA is depolymerized by an internal transesterification to form nucleotide diester intermediates which in turn are hydrolyzed to monophosphate nucleotides.

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[17] L. Vandendriessche, Acta. Chem. Scand., 7, 699 (1953).

[18] R. Markham and J. D. Smith, Nature, 168, 406 (1951).

[19] R. Markham and J. D. Smith, Biochem. J., 52, 552 (1952).

[20] R. Markham and J. D. Smith, ibid., 558 (1952).

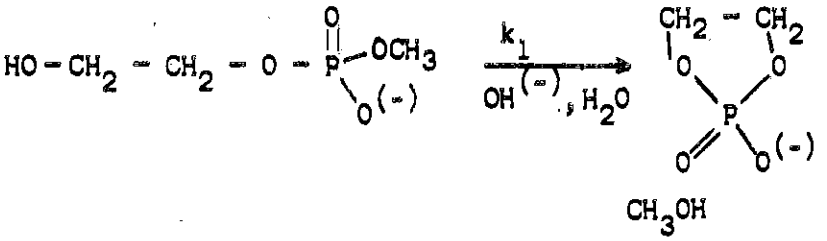
[21] D. M. Brown and A. R. Todd, J. Chem. Soc., 2708 (1952).

[22] D. M. Brown and A. R. Todd, ibid., 2715 (1952).

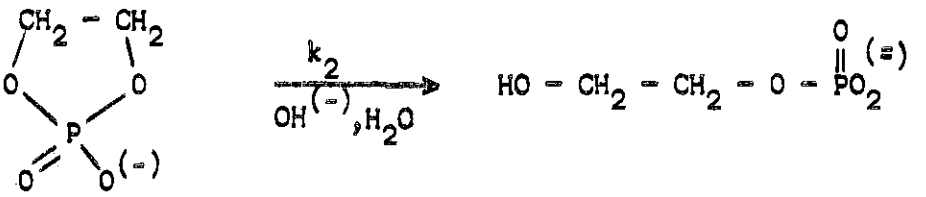
[22a] Bovine pancreatic ribonuclease is specific for the pyrimidine nucleotides. Purine nucleotides do not act as substrates for it.

Model Systems

The intermediate cyclic diester is of considerable interest because it is a reactive diester of phosphoric acid. As has already been pointed out normal phosphate diesters are unreactive when treated with aqueous base. This anomaly stimulated more detailed research on five-membered cyclic phosphates. Cox, Westheimer and Kumamoto succeeded in synthesizing the simplist such cyclic ester, ethylene phosphate. This compound was shown to be hydrolyzed in basic solution about 10^7 times more rapidly than its acyclic analog, dimethyl phosphate. This reaction is first-order in hydroxide ion and in substrate.[23]



(Eq. 1)



(Eq. 2)

One of the reasons for the great reactivity of the five-membered cyclic phosphate is a ring strain of about 5.5 kcal./mole. This value was obtained by measuring the difference in the heats of hydrolysis between ethylene methyl phosphate and β-hydroxyethyl dimethyl phosphate.[24]

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[23] J. Kumamoto, J. R. Cox, Jr. and F. H. Westheimer, J. Am. Chem. Soc., 78, 4858 (1956).

[24] J. R. Cox, Jr., R. E. Wall and F. H. Westheimer, Chem. Ind. (London), 929 (1959).

A number of phosphate esters with hydroxyl groups located close to the phosphoryl group have been synthesized and studied kinetically. These compounds would be expected to hydrolyze by a reaction pathway similar to that of RNA. The cis and trans-2-hydroxycyclohexyl benzyl phosphates were prepared and their reaction studied in an alkaline solution.[25] The trans form hydrolyzes about half as fast as the cis isomer although both compounds react much more readily than dialkyl phosphates. The cis form apparently allows easier formation of a ring which is less strained than that of the trans isomer.

Reactions involving other phosphate diesters with an adjacent hydroxyl available to attack the phosphoryl phosphorus have been performed.[26] The benzyl, methyl and glyceryl esters of cis-2-hydroxy-cyclohexyl phosphate are all hydrolyzed rapidly in alkaline solution to yield cis-2-hydroxycyclohexyl phosphate. These compounds react in the order benzyl < methyl < glyceryl. This indicates that the ring-forming step of the reaction involving phosphorus-oxygen bond breaking is rate-controlling because each of the three esters produce the same cyclic diester intermediate. Similar results were obtained using the methyl and phenyl esters of 2-hydroxypropyl phosphate.[27]

In all the cases described above the reaction was found to be first-order in hydroxide ion and in substrate. The k_1/k_2 ratio of less than 1 found in these reactions is in contrast, then, to that in the case of RNA.

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[25] D. Brown and H. M. Higson, J. Chem. Soc., 2034 (1957).

[26] D. Brown, G. E. Hall and H. M. Higson, J. Chem. Soc., 1376 (1958).

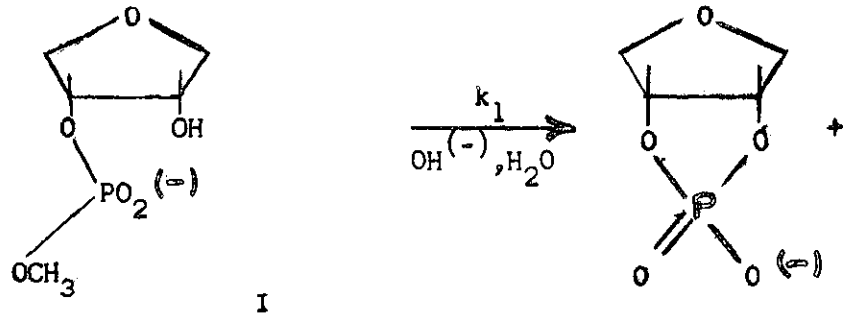
[27] D. M. Brown and D. A. Usher, Proc. Chem. Soc., 311 (1963).

RNA, therefore, in alkaline solution reacts to form a highly reactive five-membered phosphate ring more rapidly than the ring can react to form product. This presents an interesting situation and also provides an opportunity to learn more about the mode of action of ribonuclease in depolymerizing ribonucleic acid. It should be recalled that the alkaline hydrolysis of RNA appears to occur by approximately the same pathway as the hydrolysis catalyzed by ribonuclease, the chief difference being the relative magnitudes of the rate constants for the two individual steps.

It is proposed in this work that the reason for the described contrast in hydrolytic behavior between RNA and the model compounds described above is an entropy advantage caused by the geometry of the pentose ring of RNA. The favorable geometry is due to a dihedral angle of almost zero, between the β-hydroxyl group and the phosphoryl group at the 2' - and 3' - positions. The hydroxyl group is held by this structure in a favorable position to attack the phosphoryl phosphorus. All the other model compounds discussed above either allow free rotation about the carbon-carbon bond which joins the hydroxyl and the phosphoryl, or hold the reacting groups cis or trans on a cyclohexyl ring. The dihedral angle in question, even in the more favorable cis form of the cyclohexyl ring, is approximately 60°.

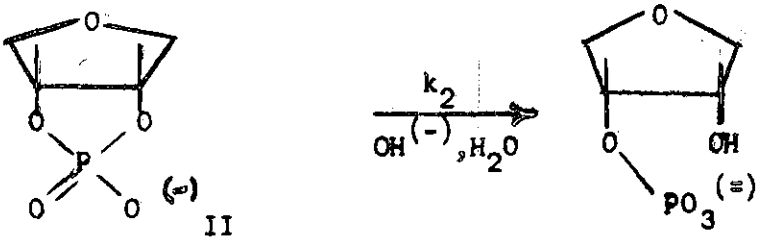
It is proposed to synthesize a β-hydroxyl phosphate diester which duplicates the geometry of the five-membered pentose ring of D-ribose, and to compare its rate of alkaline hydrolysis to that of a model of the cyclic intermediate, which also contains a D-ribose-like ring. To achieve this end, cyclohexylammonium cis-3-hydroxy-4-tetrahydro-

furanyl methyl phosphate (I) and cyclohexylammonium cis-3, 4-tetrahydrofuranyl cyclic phosphate(II) have been prepared and the rates of their reaction (Equations 3 and 4) in sodium hydroxide solution studied.



(Eq. 3)

I



(Eq. 4)

II

The ratio of k_1/k_2 obtained, will then be compared to that of the other models and an assessment of the importance of the D-ribose ring geometry to the relative magnitudes of k_1 and k_2 will be made.

CHAPTER II

EXPERIMENTAL

Instrumentation

All infrared spectra were taken on the Perkin-Elmer Infracord spectrophotometer and were calibrated with the 6.23 μ band of polystyrene. All pH measurement were made with the Beckman model G pH meter.

Synthetic

meso-Erythritol

meso-Erythritol was prepared by the method of Reppe.[28] cis-2-Butene-1,4-diol, 246.0 g., (3.0 mole), water, 250.0 g., and one half g. of osmium tetroxide were mixed and cooled to 0°. An aqueous solution of 290 ml. of a 35 percent solution of hydrogen peroxide (3.0 mole) was added to this mixture over a period of four hours with stirring; the temperature was kept between 0 and 10°. The orange reaction mixture was treated with ca. five g. of zinc dust to reduce the osmium oxides and filtered under reduced pressure. The resulting solution was concentrated on a rotary evaporator at 35° until crystals of meso-erythritol appeared. This mixture was worked up by cooling and adding an equal volume of methanol. Several crops of white crystals were obtained. The meso-erythritol was recrystallized from water by the addition of an equal volume of methanol to give 150.0 g. (48.0%) of pure white crystals,

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[28] W. Reppe and coworkers, Ann., 596, 137 (1955).

m. p. 119-120° (lit.,[28] 120°).

cis-3,4-Dihydroxytetrahydrofuran[29]

A mixture of 104.5 g. (1.0 mole) of meso-erythritol and 1.7 g. of p-toluenesulfonic acid was heated at 100° under reduced pressure (0.5 mm.). A clear, colorless oil distilled directly from the reaction mixture. This liquid was redistilled under vacuum to yield 65.0 g. (75.0%) of cis-3,4-dihydroxytetrahydrofuran, b.p. 119-120° (0.60 mm.). (lit.,[29] b.p. 118° at 0.5 mm.).

Methyl Phosphorodichloridite

This compound was prepared by the general method of Malowan, Martin and Pizzolato.[30] To 275.0 g. (2.0 mole) of phosphorus trichloride cooled in an ice-salt bath was added 64.0 g. (2.0 mole) of methanol with vigorous stirring. The resulting reaction mixture was fractionated through a 100 cm., vacuum-jacketed column packed with glass helices to yield 88.0 g. (33.0%) of methyl phosphorodichloridite, b.p. 90-92° at atmospheric pressure (lit.,[31] $b.p._{758}$ 95-96°).

Methyl cis-3,4-Tetrahydrofuranyl Cyclic Phosphite

A mixture of 190.0 g. (0.20 mole) of cis-3,4-dihydroxytetrahydrofuran and 60.0 g. (0.40 mole) of triethylamine was placed in 200.0 ml. of dry ether and cooled in an ice-salt bath. To this, 25.0 g.

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[29] C. M. Himel and L. O. Edmonds, U. S. Patent 2,572,566 (1951). [C. A., 46, 6157 (1952)].

[30] J. E. Malowan, D. Martin and P. T. Pizzolato, in "Inorganic Syntheses," Vol. 2, McGraw-Hill Inc., Maple Press Co., York, Pa., (1953).

[31] Kosolapoff, p. 199.

(0.20 mole) of methyl dichlorophosphite, dissolved in 150 ml. of dry ether, was added slowly and with vigorous stirring. The triethylamine hydrochloride formed was removed by filtration under reduced pressure and the filter cake washed several times with dry ether. These washings were added to the reaction solution and stripped on a rotary evaporator. A clear yellow liquid resulted which upon distillation under vacuum yielded 13.1 g. (47.0%) of a clear colorless liquid, b.p. 67-68° (1.0 mm.).

Methyl cis-3,4-Tetrahydrofuranyl Cyclic Phosphate.

The phosphite was oxidized by the method of Cox.[32] Twenty-six g. (0.14 mole) of cis-3,4-dihydroxytetrahydrofuranyl methyl phosphite was cooled in an ice-salt bath to -10°. Into this liquid NO_2 gas was bubbled until a greenish color appeared. The reaction mixture was distilled under vacuum and yielded 14.6 g. (71.4%) of the desired phosphate, b. p. 135-138° (0.10 mm.). This material formed large needles after standing at room temperature in a sealed container several months.

Cyclohexylammonium cis-3,4-Tetrahydrofuranyl Cyclic Phosphate[33]

10.4 g. (0.10 mole) of meso-erythritol and 9.8 g. (0.10 mole) of concentrated phosphoric acid were heated at around 125° for 75 hours. The resulting brown, amorphous solid reaction mixture was shaken with cold absolute ethanol to dissolve any reaction by-products. The ethanol-

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[32] J. R. Cox, Jr. and F. H. Westheimer, J. Am. Chem. Soc., 80, 5441 (1958).

[33] P. Carré, Ann. Chim., [8], 5, 385 (1902).

insoluble cis-3,4-tetrahydrofuranyl cyclic phosphoric acid was isolated by gravity filtration and washed five times with small amounts of dry ether. The fine tan powder was treated with an excess of cyclohexylamine and the reaction mixture was cooled.

The salt was dissolved in cold n-butanol. Treatment of this solution with dry ether produced a fine white powder which was washed several times with dry ether and dried under vacuum. Three g. (11.3%) of the cyclohexylammonium cis-3,4-tetrahydrofuranyl cyclic phosphate were thus collected.

The infrared spectrum of this material was found to be identical with that of an anthentic sample.[34]

Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Methyl Phosphate

Twenty-one g. (0.12 mole) of methyl cis-3,4-tetrahydrofuranyl cyclic phosphate was dissolved in water, cooled to -10° by means of an ice-salt bath, and titrated with a 20.0 percent by weight cyclohexylamine aqueous solution. The pH changed from 1.0 to 10.0 during the addition. The resulting solution was frozen with Dry ice-acetone and lyophilized. Thirty-two and one half g. of a granular yellow solid was obtained. Several recrystallizations from ethanol-ether yielded 3.70 g. (7.3%) of while needles.

Barium cis-3-Hydroxy-4-tetrahydrofuranyl Phosphate

One fourth g. (0.00068 mole) of bis-cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl phosphate was dissolved in about five drops

[34]The authentic sample was prepared by Mr. J. Farmer by ring closure of the trans bromohydrin monophosphate.

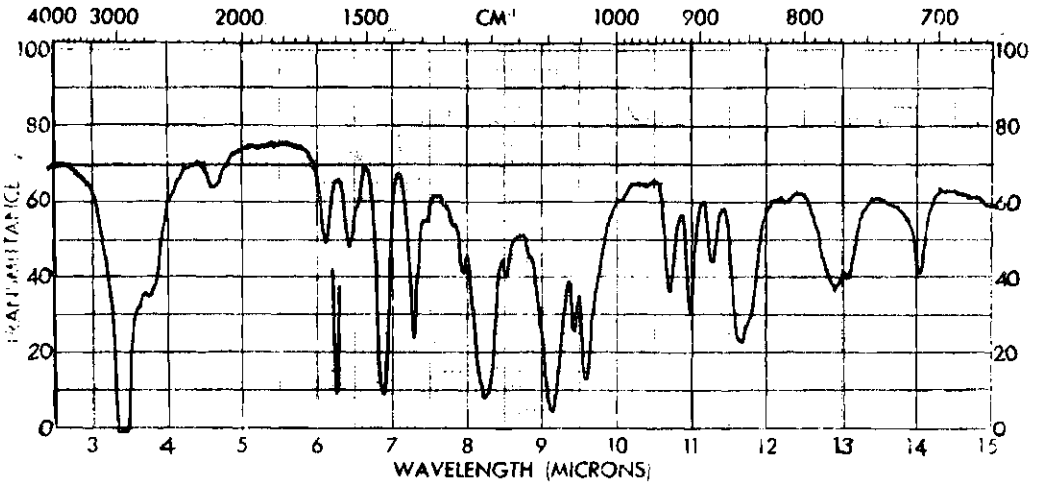

Figure 2. Infrared Spectrum of an Authentic Sample of Cyclohexylammonium cis-3,4-Tetrahydrofuranyl Cyclic Phosphate

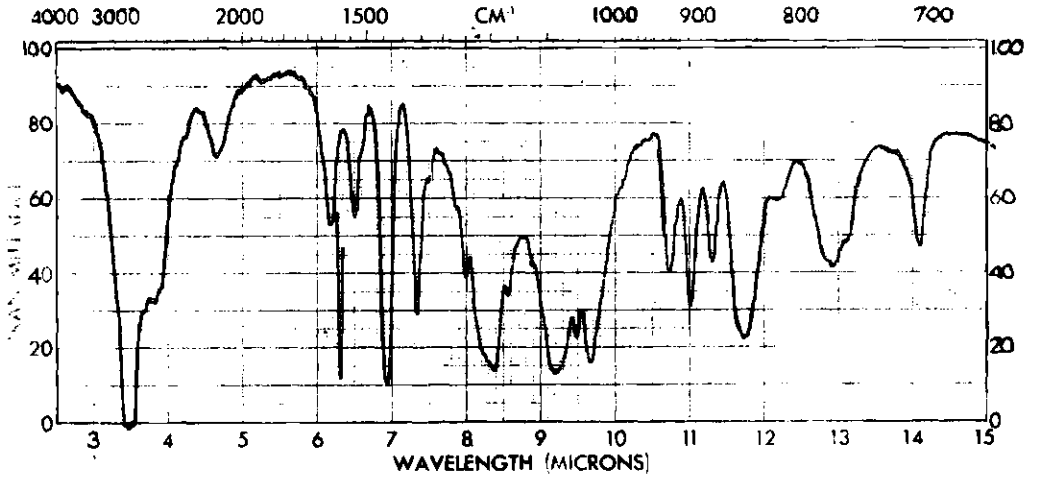

Figure.3. Infrared Spectrum of Cyclohexylammonium cis-3,4-Tetrahydrofuranyl Cyclic Phosphate from the **Neutralization** of the **Reaction** Mixture of meso-Erythritol and **Phospheric** Acid

of 50 percent ethanol-water. To this solution was added 10.0 ml. of a saturated barium chloride aqueous solution. This solution was diluted with 50.0 ml. of ethanol, producing a white gelatinous precipitate. This material was recrystallized from water by addition of several volumes of ethanol and then digested to facilitate filtration. This method produced from the jelly 0.05 g. (23.0%) of fine white powder.

Kinetics

The kinetics of the hydroxide ion-catalyzed hydrolysis of cyclohexylammonium cis-3,4-tetrahydrofuranyl cyclic phosphate and cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl phosphate were studied by a titration method,[35] involving the measurement of the rate of appearance of the cis-3-hydroxy-4-tetrahydrofuranyl phosphate dianion. The endpoints for the titration of the primary and secondary acid ionizations occur at pH 4.3 and pH 8.0 respectively in solutions of ionic strength equal to one. The procedure is as follows: 50.0 ml. of standard sodium hydroxide solution, made up to an ionic strength of one with sodium perchlorate, was placed in a polyethylene bottle and brought up to 60.0° in a constant temperature bath. Approximately 150 mg. of the sample salt was added and 5.00 ml. samples withdrawn with a pipette at known time intervals. These samples of reaction solution were quenched immediately in enough 0.1 N hydrochloric acid at 0° to bring the pH to approximately 10. The pH was then adjusted to 8.0 and the volume of standard 0.01 N hydrochloric acid solution required to change the pH from 8.0 to 4.3 measured.

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[35] J. Kumamoto, J. R. Cox, Jr., and F. H. Westheimer, J. Am. Chem. Soc., 78, 4858 (1956).

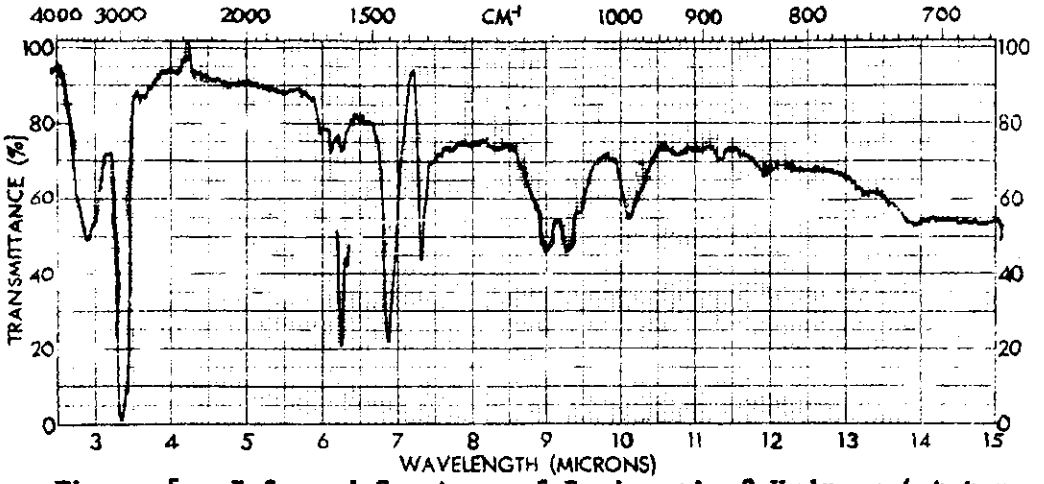

Figure 5. Infrared Spectrum of Barium cis-3-Hydroxy-4-tetrahydrofuranyl Phosphate from the Hydrolysis of Methyl cis-3,4-Tetrahydrofuranyl Cyclic Phosphate

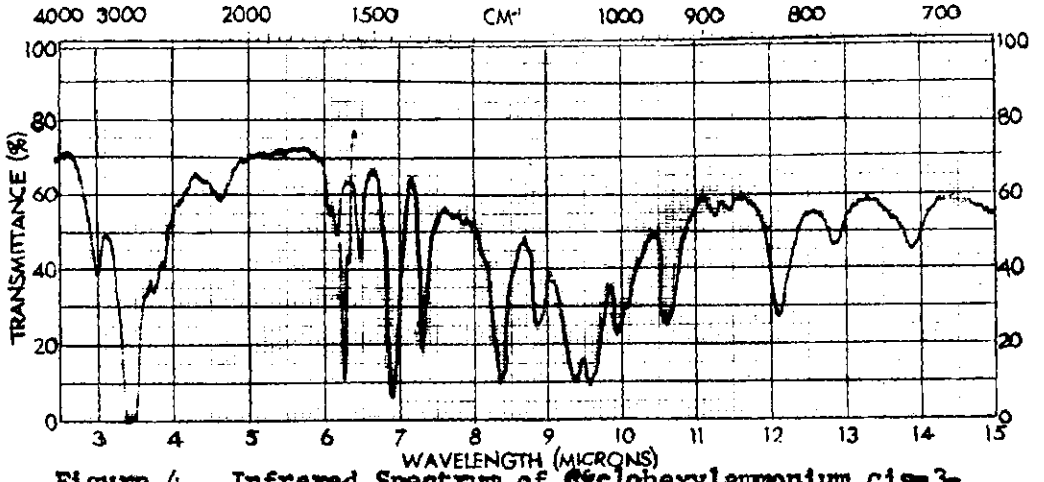

Figure 4. Infrared Spectrum of Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Methyl Phosphate from the Reaction of Methyl cis-3,4-Tetrahydrofuranyl Cyclic Phosphate with Water and Cyclohexylamine

Bath temperatures were held constant to within ± 0.05°.

Product Analysis

The products of the base-catalyzed hydrolysis of the two cyclohexylammonium diesters were studied as follows: a weighed quantity of the diester monoanion salt was placed in 5.0 ml. of water with an equimolar amount of barium hydroxide octahydrate. This solution was heated overnight at 60.0°. The reaction mixture was then worked up by adding 30.0 ml. of ethanol and digesting the resulting gelatinous material until it became a fine powder. This was recrystallized once from ethanol-water and isolated by filtration under reduced pressure.

Determination of the Endpoints for Titration Between the First and Second Acid Ionizations of bis-Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Phosphate

A sample of this monophosphate ester salt was placed in a 1.0 N sodium hydroxide solution. The solution was titrated with a 1.0 N HCl using a pH meter. The pH vs. volume of 1.0 N HCl data were plotted. The two endpoints were observed at pH 4.3 and pH 8.0 and correspond to endpoints of the first and second acid ionization of this phosphomonoester.

Treatment of the Experimental Data

Calculations

The rate equation (Eq. 1) which describes the alkaline catalyzed hydrolysis of cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl methyl phosphate is in general form that of two consecutive pseudo-first order reactions with rate constants of comparable magnitude.[36]

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[36] A. A. Frost and R. G. Pearson, in "Kinetics and Mechanism," 2nd ed., John Wiley and Sons, Inc., New York, New York, 1960, p. 167.

$$C = A_0\left[1 + \frac{1}{k_1 - k_2}\left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)\right] \qquad \text{(Eq. 1)}$$

Where C is the concentration of dianion formed at time t, A_o is the initial concentration of the diester and k_1 and k_2 are the rate constants for the first and second steps of the reaction respectively.

Even with t and all the constants known, it proved impossible to solve this expression explicitly for k_1. Because of this, a trial and error solution was attempted but this method involved long and tedious calculations. Equation (5) was solved for k_1 by use of a Burroughs B-5000 computer. The program used instructed the computer to perform a trial and error analysis of the experimental data from each kinetic run.[37] The computer was instructed to use equation (Eq. 5) to calculate C's using k_2, experimental t's and a carefully selected range of values of k_1, changing k_1 in steps of 0.001 min.$^{-1}$. The computer then compared these calculated C's with the corresponding experimental C's and calculated a standard error from the differences. The value of k_1, giving C's with a minimum standard error was chosen as the experimental k_1, for each kinetic run.

Correction for bis-Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Phosphate in Kinetic Substrates

bis-Cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl phosphate proved to be a difficult impurity to remove from both cyclohexylammonium phosphodiester compounds without large losses of the desired diester salts.

[37]This program was suggested by Mr. M. Roberts, whose aid is hereby gratefully acknowledged.

It is believed that the dianion has no effect on the rates of reaction being studied. It was necessary however, to determine the percentage of dianion present in each salt so that kinetic calculations could be corrected when necessary. To determine this, a weighed sample of each preparation of the diester salt was dissolved in a 0.001 N sodium hydroxide solution and titrated quickly with standard 0.01 N hydrochloric acid using a pH meter. The resulting pH vs. volume of 0.01 N HCl added curve showed breaks at pH 8.0 and 4.3. The volume of 0.01 N HCl required to titrate between pH 8 to pH 4.3 gives a direct measure of the amount of dianion present. The cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl methyl phosphate contained 10.0 percent by weight, bis-cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl phosphate. The cyclohexylammonium cis-3,4-tetrahydrofuranyl cyclic phosphate contained 7.0% by weight of this dianion salt.

CHAPTER III

RESULTS

Kinetics

Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Methyl Phosphate

This phosphodiester decomposes in aqueous base in two consecutive pseudo-unimolecular steps which have rate constants of comparable magnitude. The general form of this hydrolysis is as follows:

$$A \xrightarrow{k_1} B \qquad \text{(Eq. 1)}$$

$$B \xrightarrow{k_2} C \qquad \text{(Eq. 2)}$$

This gives the following three differential rate equations:

$$\frac{dA}{dt} = -k_1 A \qquad \text{(Eq. 3)}$$

$$\frac{dB}{dt} = k_1 A - k_2 B \qquad \text{(Eq. 4)}$$

$$\frac{dC}{dt} = k_2 B \qquad \text{(Eq. 5)}$$

Equation 3 integrates by normal means to yield the following:

$$A = A_o e^{-k_1 t} \qquad \text{(Eq. 6)}$$

Where A_o is the initial concentration of compound A, A is the concentration of compound A at time t. Substituting this into Equation

4 yields Equation 7.

$$\frac{dB}{dt} = k_1 A_o e^{-k_1 t} - k_2 B \qquad \text{(Eq. 7)}$$

This equation may be integrated to give the following:

$$B = \frac{A_o k_1}{k_2 - k_1}\left(e^{-k_1 t} - e^{k_2 t}\right) \qquad \text{(Eq. 8)}$$

Where B is the concentration of compound B at anytime t.

$$A + B + C = A_o \qquad \text{(Eq. 9)}$$

Rearranging Equation 9 give the following equation:

$$C = A_o - A - B \qquad \text{(Eq. 10)}$$

Substituting Equations 6 and 8 for A and B in Equation 10 gives the following equation:

$$C = A_o\left[1 + \frac{1}{k_1 - k_2}\left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)\right] \qquad \text{(Eq. 11)}$$

This equation would give curves of the types seen in Figure 9.[38]

A sample plot of the experimental data obtained in this work appears in Figure 8. The C curves in Figures 8 and 9 suggest that the compound being studied is reacting in the manner described above.

Equation 11 was solved by use of a trial and error computer program and the following pseudo-unimolecular rate constants were

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[38] Frost and Pearson, pp. 166-167.

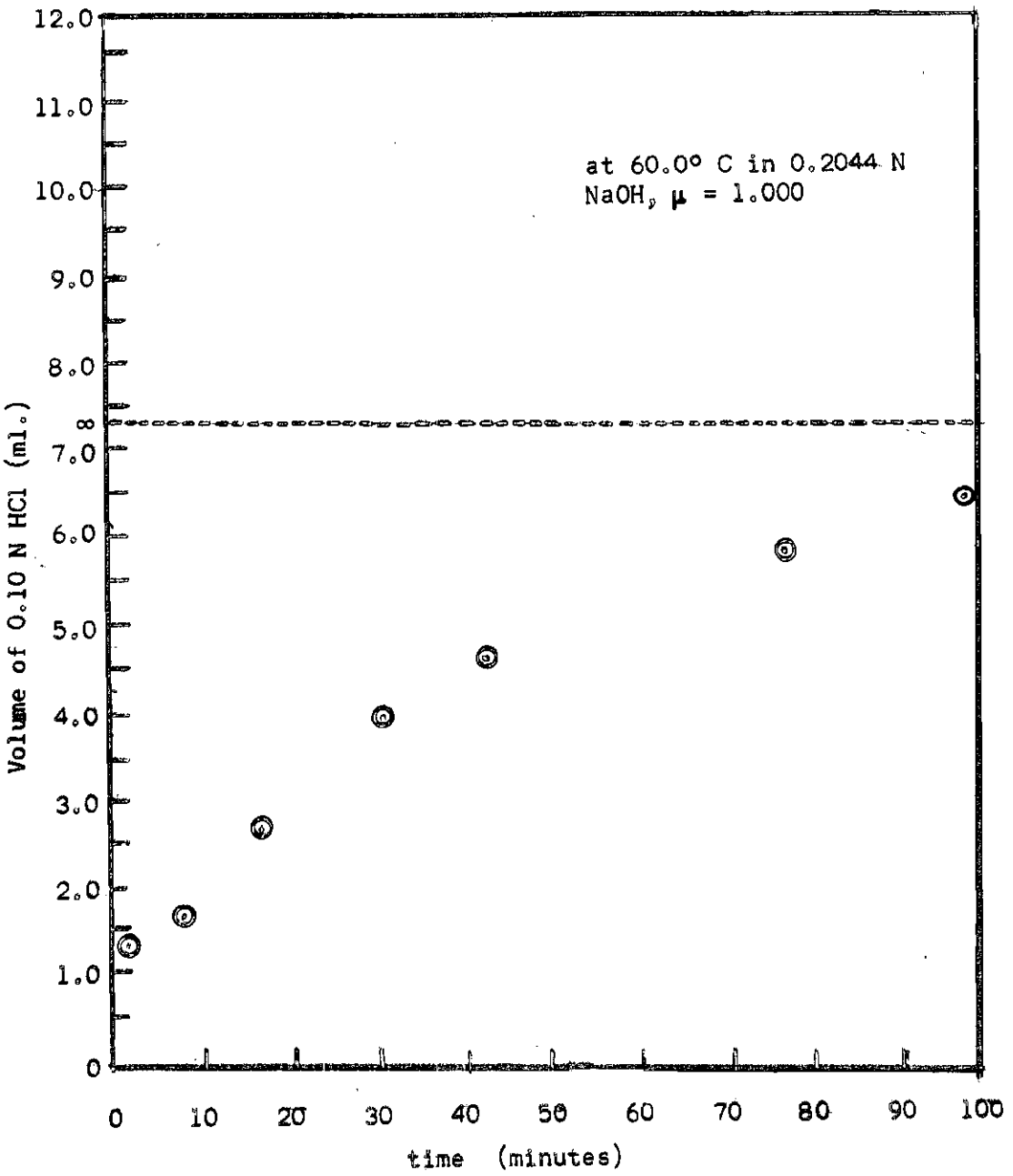

Figure 8. Plot of the Rate of Formation of cis-3-Hydroxy-4-tetrahydrofuranyl Dianion from Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Methyl Phosphate.

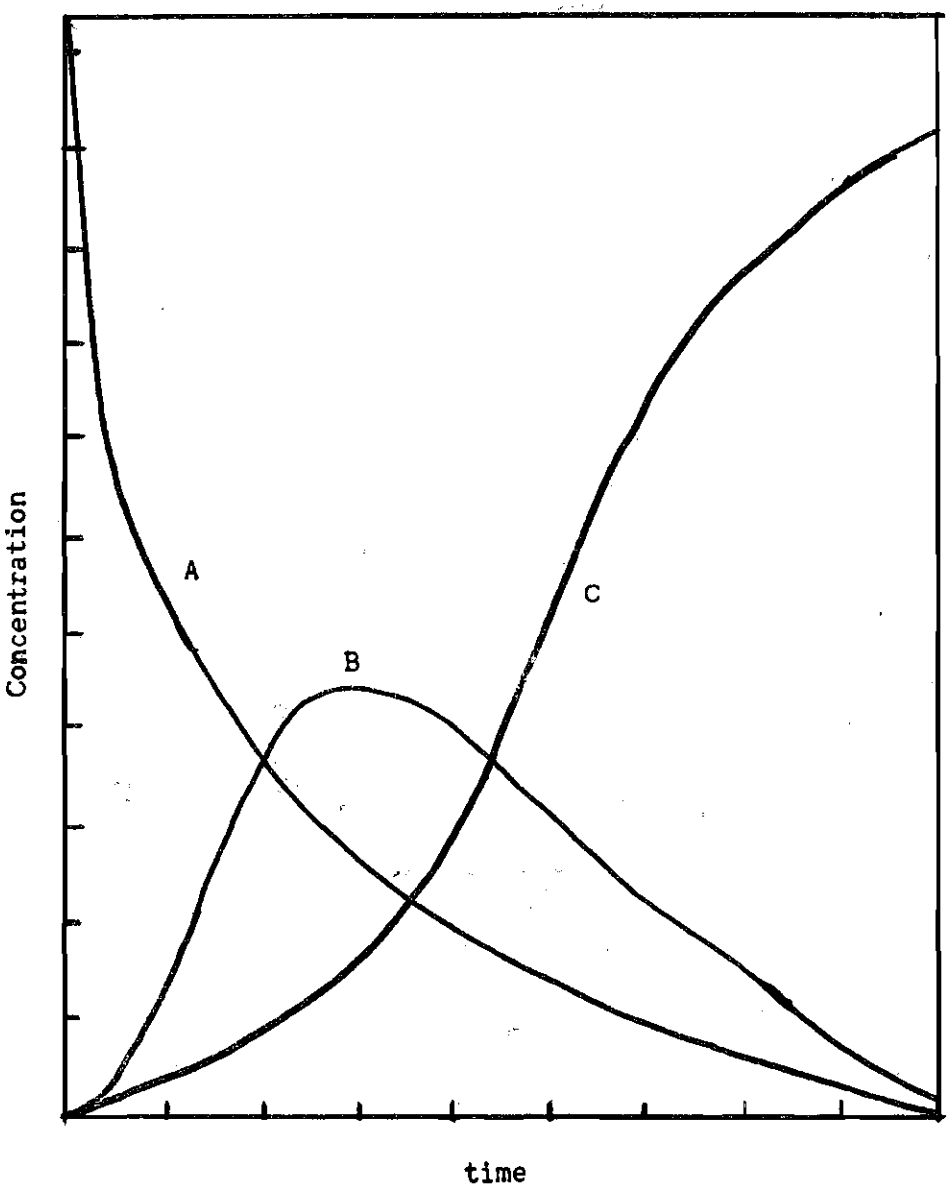

Figure 9. Concentration-time Curves for A, B, and C in Series First-order Reactions.

obtained; $k_1 = 0.116 \pm 0.009$ min.$^{-1}$ in 0.1008 N sodium hydroxide at 60.0° with an average standard error for each kinetic run of 1.3×10^{-3}. In order to show that the reaction is first-order in hydroxide ion, it was run in 0.2044 N sodium hydroxide at the same temperature. A $k_1 = 0.171 \pm 0.014$ min.$^{-1}$ was obtained. The standard error for each kinetic run was 4.6×10^{-4}. The ratio of base strengths was 2.03 while the ratio of rate constants in the two solutions was only 1.47. These data indicate only a rough first-order kinetic dependence upon hydroxide ion. However, there are two factors to be considered. Firstly, an experimental error of about eight percent is inherent in the experimental procedure used. Also, bringing the ionic strength up to one with sodium perchlorate is but a crude correction. The Debye-Hückel theory does not hold well in solutions as concentrated as these.

The presence of bis-cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl phosphate in the starting material was corrected for in the computer program used to solve the rate equation.

Cyclohexylammonium cis-3,4-tetrahydrofuranyl Cyclic Phosphate

The pseudo-unimolecular reaction of the general type

$$B \xrightarrow{k_2} C \qquad \text{(Eq. 12)}$$

is described by Equation 13.

$$\frac{dB}{dt} = k_2[B] \qquad \text{(Eq. 13)}$$

When Equation 13 is integrated by normal means between the limits, $B = B_o$ when $t = 0$ and $B = B$ when $t = t$, it gives Equation

14.[39]

$$\log_{10} \frac{B}{B_o} = \frac{-k_2}{2.303} t \qquad \text{(Eq. 14)}$$

Where B is the concentration of compound B at time t, B_o is the concentration of compound B at time = 0 and k_2 is the pseudo-unimolecular rate constant for this reaction. If we let X equal the amount of C formed at time t and assuming 100 percent reaction of B to form C, $X_\infty = B_o$, then $B = X_\infty - X$. Substituting this into Equation 14, Equation 15 is obtained.

$$\log_{10} \frac{X_\infty - X}{X} = \frac{k_2}{2.303} t \qquad \text{(Eq. 15)}$$

X and X_∞ are the experimentally determined volumes of 0.01 N HCl necessary to titrate the dianion phosphate ester produced at times t and infinity, respectively. Plotting of $\log_{10} (X_\infty - X)$ against t should give a straight line of slope $-k_1$. A straight line was obtained and at 60.0° the pseudo-unimolecular constant was $k_2 = 0.0504 \pm 0.0014$ min.$^{-1}$ in 0.1003 N sodium hydroxide solution. In order to show that this reaction is first order in hydroxide it was run at the same temperature but in a 0.2000 N sodium hydroxide. A pseudo-unimolecular rate constant of $k_2 = 0.121 \pm 0.001$ min.$^{-1}$ was obtained. The ratio of base strengths is 1.99 whereas the ratio of rate constants is 2.47. This of course is not a strict second order rate dependency upon hydroxide but taking into consideration the same two factors as were discussed in

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[39] Frost and Pearson, pp. 12-13.

the case of the cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl methyl phosphate this ratio is satisfactory. The experimental error involved in the technique used to obtain these rate constants is about five percent.

A plot of the reaction data; the volume of 0.01 N HCl vs. time, gives a simple exponential curve of the expected shape (Figure 10). The concentration of C (the dianion salt formed) begins at zero and decreases more and more slowly as it approaches infinity.

A sample plot of the $\log_{10}$ $(X_\infty - X)$ vs. time is included (Figure 11).

Any dianion impurity present in the cyclohexylammonium cis-3-hydroxy-4-tetrohydrofuranyl phosphate would have no effect on the rate constant obtained because it is accounted for in both X_∞ and X and would be removed when the difference is taken.

Product Analyses

The cyclohexylammonium cis-3-hydroxy-4-tetrahydrofuranyl methyl phosphrate gave a 60.7 percent yield of the barium salt of the dianion whereas the cyclohexylammonium cis-3,4-tetrahydrofuranyl cyclic salt gave 48.0 percent yield of the barium salt.

Both salts studied kinetically were expected to yield the same product. The infrared spectra in Nujol nulls of these products were superimposable (Figs. 6 and 7). They were also identical with the infrared spectrum of the barium salt of the cis-3-hydroxy-4-tetrahydrofuranyl phosphate obtained from the methyl cis-3,4-tetrahydrofuranyl cyclic phosphate (Fig. 5).

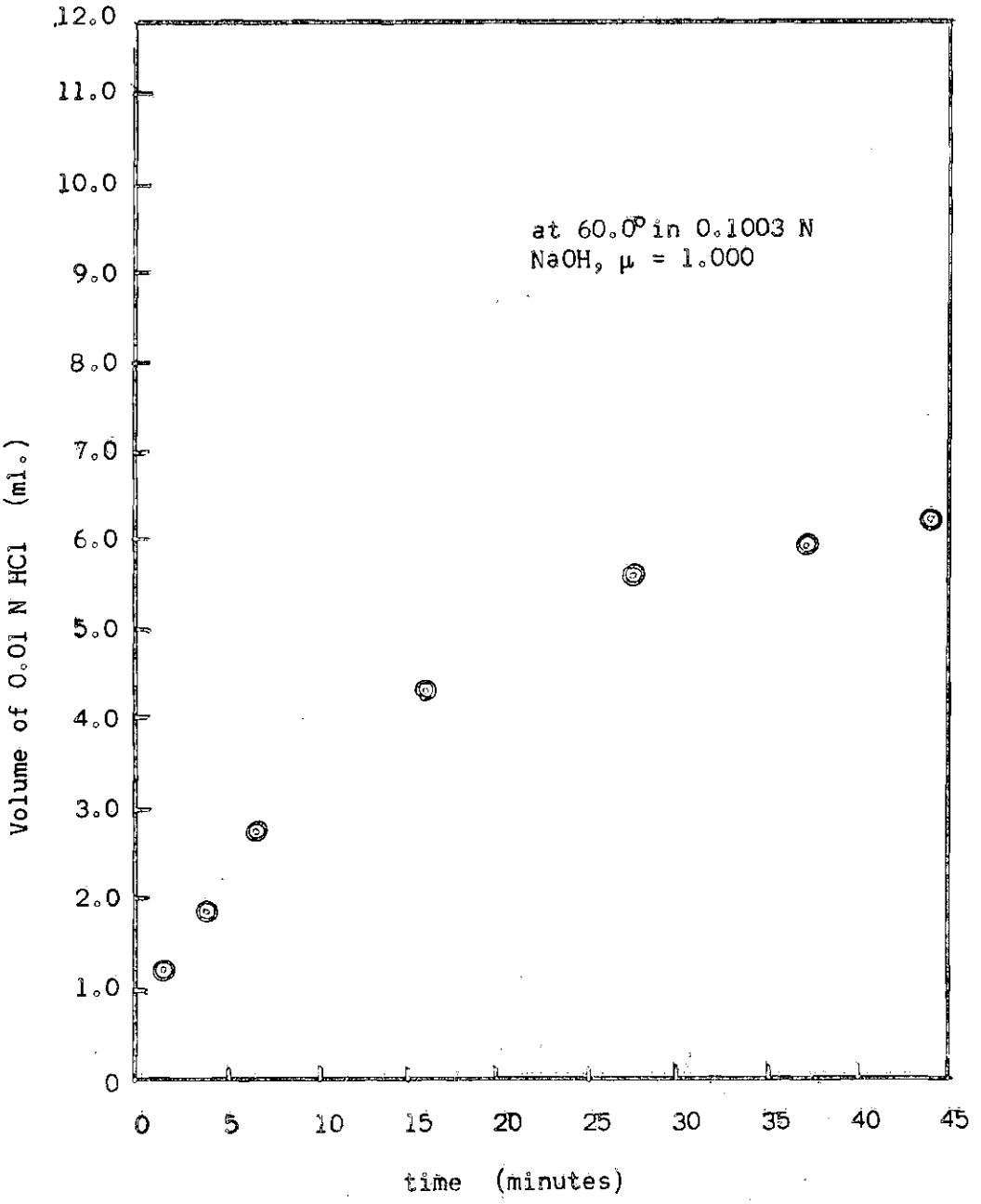

Figure 10. Plot of the Rate of Formation cis-3-Hydroxy-4-tetrahydrofuranyl Dianion from Cyclohexylammonium cis-3,4-Tetrahydrofuranyl Cyclic Phosphate.

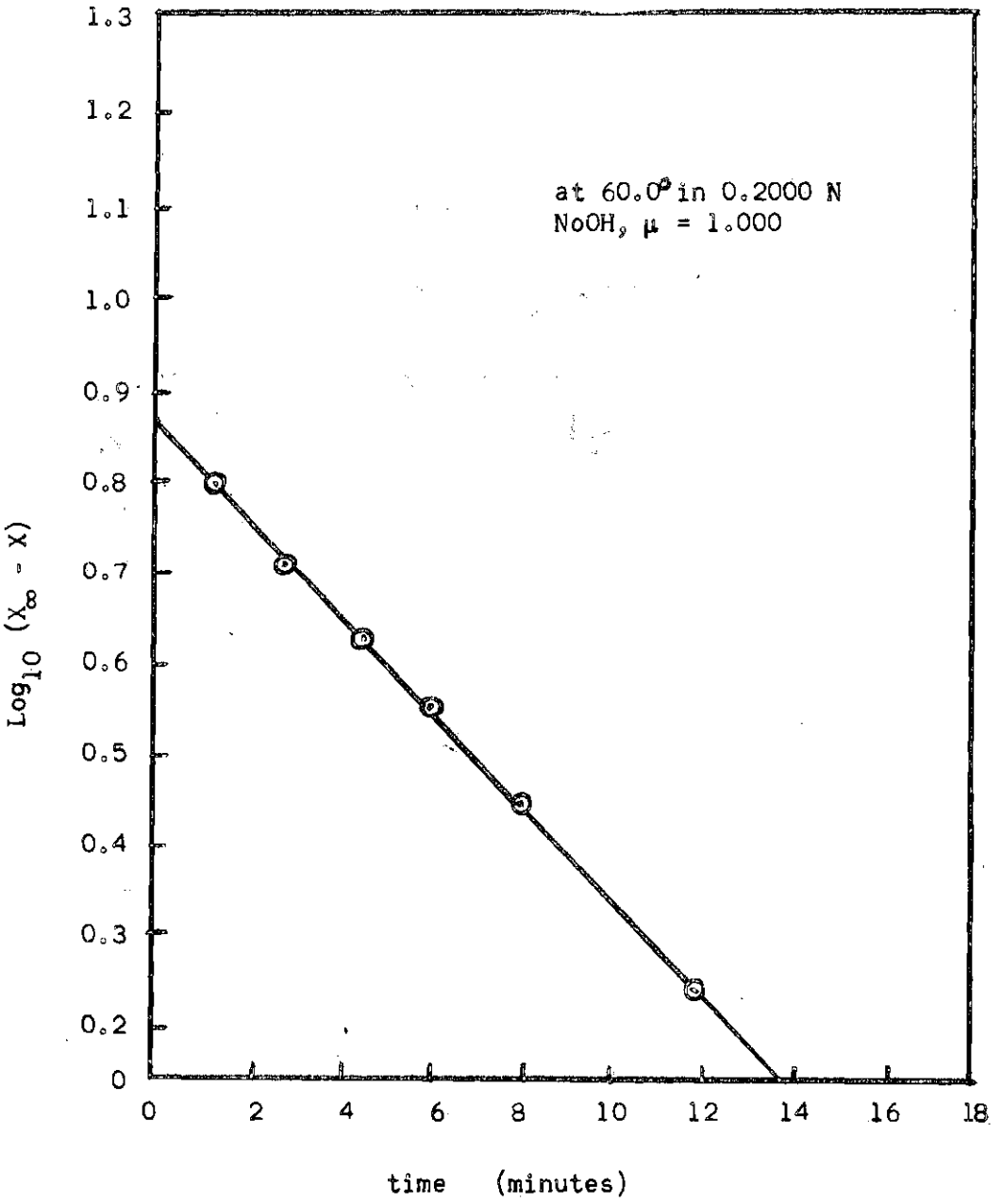

Figure 11. First-order Plot of the Data for the Base-catalyzed Hydrolysis of Cyclohexylammonium cis-3,4-Tetrahydrofuranyl Cyclic Phosphate.

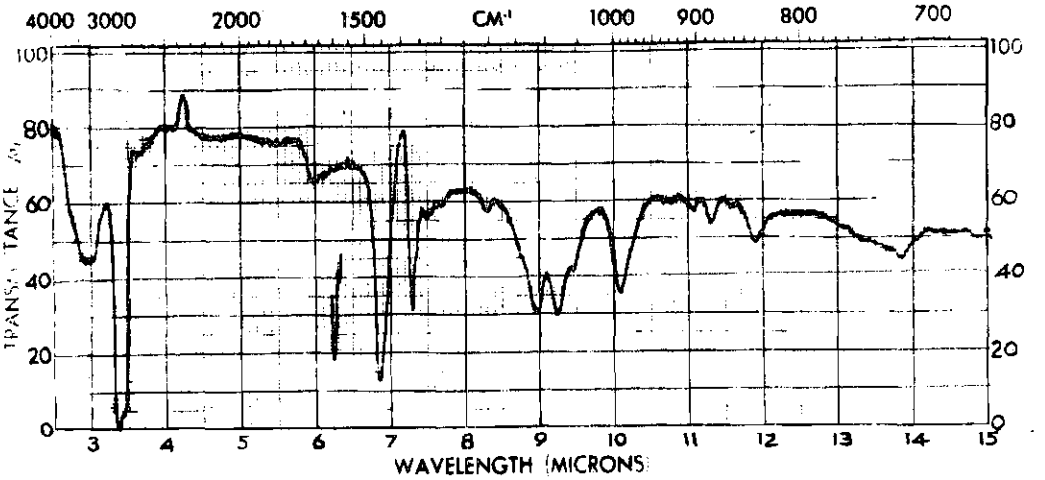

Figure 6. Infrared Spectrum of Barium cis-3-Hydroxy-4-tetrahydrofuranyl Phosphate from the Product Analysis of the Hydrolysis of Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Methyl Phosphate

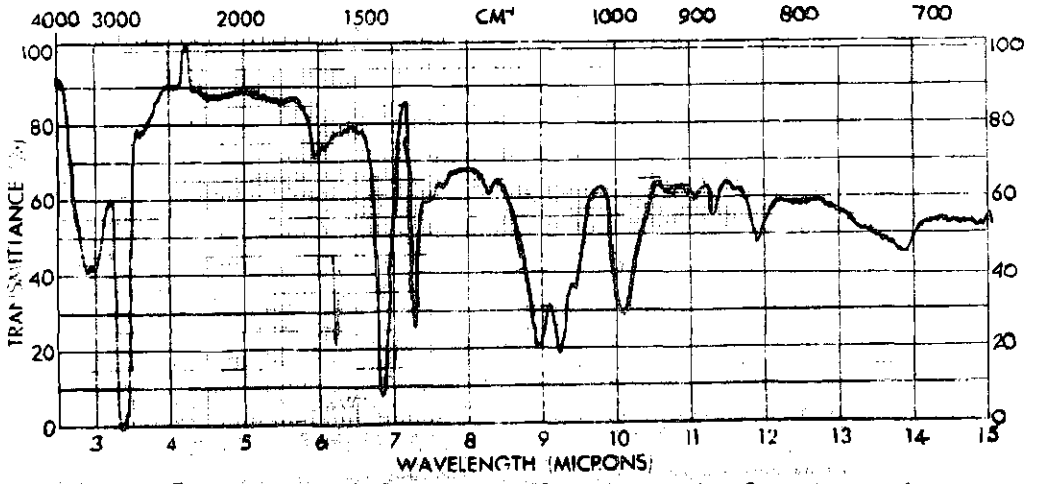

Figure 7. Infrared Spectrum of Barium cis-3-Hydroxy-4-tetrahydrofuranyl Phosphate from the Product Analysis of the Hydrolysis of Cyclohexylammonium cis-3,4-Tetrahydrofuranyl-Cyclic Phosphate

Table 1. Data for the Base-catalyzed Hydrolysis of Cyclohexylammonium cis-3-Hydroxy-4-tetrahydrofuranyl Methyl Phosphate at 60.0°.

Base Concentration	Pseudo-unimolecular k_2 min.$^{-1}$	Average values k_2 min.$^{-1}$
0.1003	0.0522	
0.1003	0.0489	0.0504 ± 0.0014
0.1003	0.0506	
0.1003	0.0522	
0.2000	0.121	
0.2000	0.119	0.121 ± 0.001
0.2000	0.123	

Ionic strength made up to one in all cases with $NaClO_4$

Table 2. Data for the Base-catalyzed Hydrolysis of Cyclohexylammonium cis-3,4-Tetrahydrofuranyl Cyclic Phosphate at 60.0°.

Base Concentration	Pseudo-unimolecular k_2 min.$^{-1}$	Standard Error	Average value k_2 min.$^{-1}$
0.1008	0.126	7.44×10^{-4}	
0.1008	0.102	1.68×10^{-3}	0.116 ± 0.009
0.1008	0.119	1.43×10^{-3}	
0.2044	0.192	4.48×10^{-4}	
0.2044	0.155	4.15×10^{-4}	0.171 ± 0.014
0.2044	0.165	5.18×10^{-4}	

Ionic strength made up to one in all cases with $NaClO_4$

CHAPTER IV

CONCLUSIONS

The following experimental results were obtained:

1. The base-catalyzed hydrolysis of both model compounds is first-order in base.

2. The rate of reaction of the model of the intermediate compound (II) is also first-order in substrate.

3. The ratio of rate constants k_1/k_2 for the base-catalyzed hydrolysis of the model system as determined in this work is approximately two.

The above results indicate that the model system studied in his work is an intermediate case, between that of RNA and the model compounds reported by other workers. The k_1/k_2 ratio in the case of RNA probably is larger than two, judging from the product isolation studies cited, but this number has not been measured kinetically. The k_1/k_2 ratio in all the model compounds reported previously is much less than one.

These results indicate that while the favorable geometry of the incipient ring members appears to give the transition state of the ring-closure step in RNA an important entropy advantage over that of the other model compounds, other factors are also important. Several of these possible effects are listed below:

1. The source of another entropy advantage the RNA hydrolysis possesses over the model systems is the entropy increase which occurs

when a polymer is broken into smaller fragments. This effect would involve the ring formation because this is the step in which the RNA polymer is broken up.

2. The RNA and the model compounds differ in several structural features, which may have an effect on the reaction rates. For instance, the nitrogenous base at the 1'-carbon could have a steric or electronic influence on the alkaline hydrolysis of RNA which would not be apparent in the model studied.

3. A factor which could be of importance is the secondary structure of the RNA polymer.

Since the activation energies of depolymerization of RNA have not been measured, it is difficult to estimate the magnitudes of such effects and to predict whether they are large enough to explain the difference in the k_1/k_2 ratios found in the alkaline hydrolyses of RNA and the model studied in this work.

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*The abbreviations used in the text and bibliography are the standard abbreviations used by the American Chemical Society's Abstracting Service.